

Short  
CommunicationComplete genome sequences of two new variants  
of *Grapevine rupestris* stem pitting-associated  
virus and comparative analyses

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*Grapevine rupestris* stem pitting-associated virus (GRSPaV), a member of the genus *Foveavirus* within the family *Flexiviridae*, is the putative causal agent of the disease *Rupestris* stem pitting (RSP) of grapevines. GRSPaV comprises a family of variants whose pathological characteristics have not been determined. Recently, many of the indicator 'St George' plants (*Vitis rupestris*) used throughout the world to index RSP tested positive for GRSPaV. This finding questions the validity of past biological indexing results. In this work, a representative genomic region of GRSPaV was first sequenced from ten 'St George' plants from two sources and it was demonstrated that nine of them carried a new variant, GRSPaV-SG1. The genomes of GRSPaV-SG1 and GRSPaV-BS from 'Bertille Seyve 5563' plants were sequenced, revealing a genome structure identical to that of GRSPaV-1. It was demonstrated experimentally that infection of 'St George' plants with GRSPaV-SG1 is asymptomatic and thus it is proposed that GRSPaV-SG1 infection should not have interfered with the outcome of past indicator indexing. This represents the first attempt to link a GRSPaV variant with pathological properties.

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*Grapevine rupestris* stem pitting-associated virus (GRSPaV) is the putative causal agent of *Rupestris* stem pitting (RSP), a component of the Rugose wood disease complex, which is widespread in grapevines (Goheen, 1988; Martelli, 1993). Through cloning of a double-stranded RNA (dsRNA) species isolated from RSP-infected grapevines, the genomes of two almost identical GRSPaV isolates were sequenced independently in two laboratories (Meng *et al.*, 1998; Zhang *et al.*, 1998). The first isolate was designated *Rupestris* stem pitting-associated virus (RSPaV) by Meng *et al.* (1998) while the second was named GRSPaV (Zhang *et al.*, 1998). To avoid confusion in nomenclature, we have used the name GRSPaV. GRSPaV is a member of the genus *Foveavirus* (Martelli & Jelkmann, 1998) within the family *Flexiviridae* (Adams *et al.*, 2004). The genome of GRSPaV possesses five open reading frames (ORFs), potentially encoding proteins involved in replication (ORF1), movement (ORF2, -3 and -4) and virion formation (ORF5) (Meng & Gonsalves,

2003). GRSPaV is widespread among different grapevine genotypes and occurs worldwide (Meng *et al.*, 1999a; Zhang *et al.*, 1998; Nolasco *et al.*, 2000; Meng & Gonsalves, 2003). Compelling evidence suggests that GRSPaV is diverse, comprising a family of sequence variants. Moreover, mixtures of GRSPaV variants are commonly detected in grapevines (Meng *et al.*, 1999b; Rowhani *et al.*, 2000; Santos *et al.*, 2003). However, the pathological properties of the different GRSPaV variants remain unknown.

*Vitis rupestris* 'St George' is the standard biological indicator used worldwide to diagnose RSP. In 1995, when we started to develop RT-PCR assays, 'St George' plants were used as negative controls. Unexpectedly, these 'St George' plants tested positive for GRSPaV. This finding was confirmed by consistent detection of GRSPaV by using RT-PCR and Western blotting in 'St George' plants obtained from two sources; among the 29 'St George' plants tested, 23 were positive for GRSPaV (Meng *et al.*, 2000, 2003). Likewise, Minafra *et al.* (2000) detected GRSPaV in the 'St George' selection maintained at the University of Bari, Italy.

This finding triggered several questions. Were these 'St

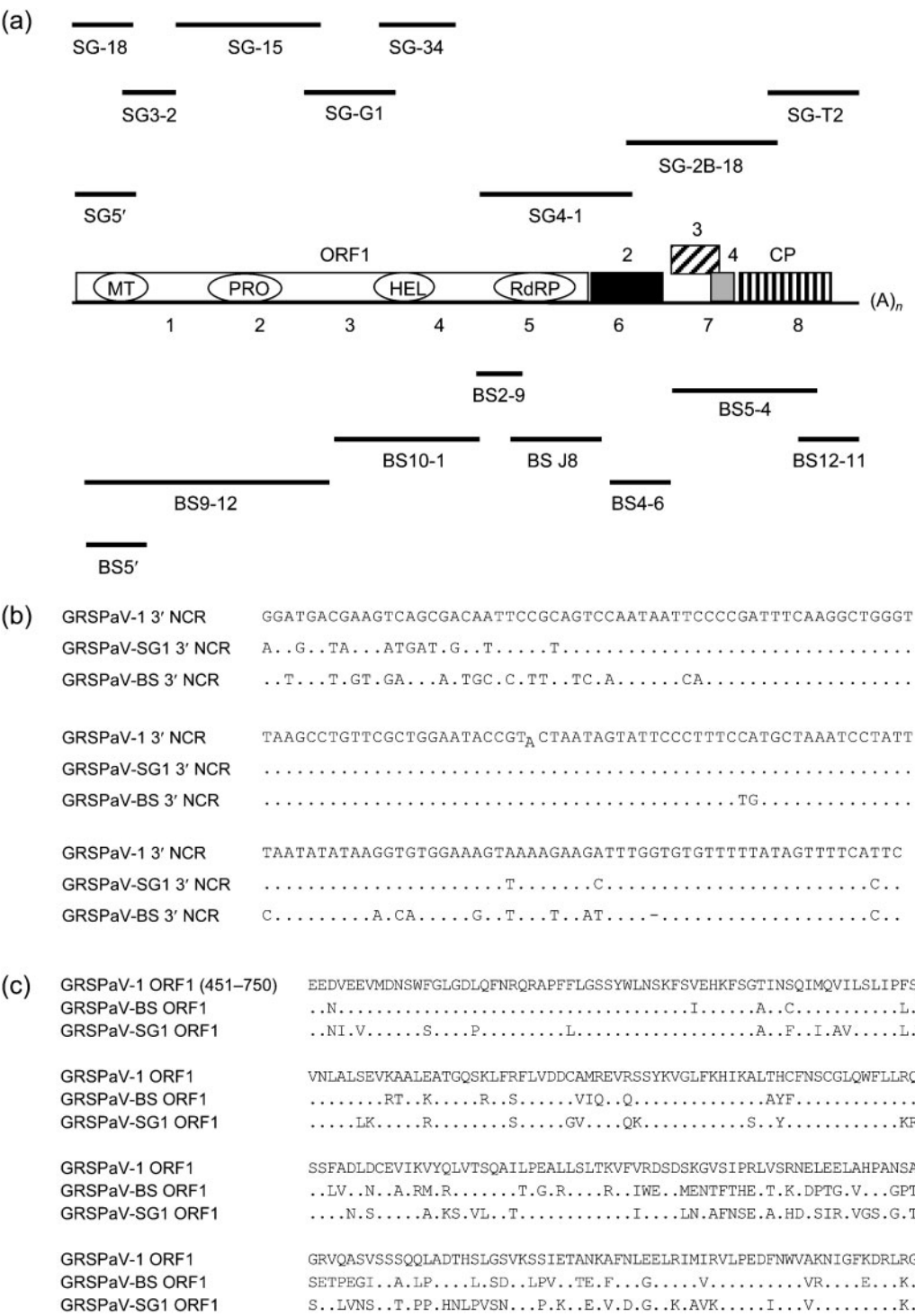
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George' plants also infected with a mixture of GRSPaV variants? Did these variants differ in genome sequence from the previously sequenced ones? Would these variants elicit RSP symptoms on the indicator 'St George' plants? Most importantly, would infection of 'St George' plants with these GRSPaV variants invalidate results of past indicator indexing experiments conducted in many countries?

To answer these questions, we first analysed the genetic

diversity of GRSPaV in 'St George' plants. dsRNAs were isolated from dormant cambium scrapings of ten 'St George' indicator plants: five from Sidney, British Columbia, Canada, and the other five from Geneva, New York, USA. As a positive control, dsRNAs isolated from French-American hybrid 'Bertille Seyve (BS) 5563' plants were also assayed. All of these plants repeatedly tested positive for GRSPaV (Meng *et al.*, 1998, 1999a, 2003). Isolated dsRNAs were reverse-transcribed by using Moloney murine



leukemia virus reverse transcriptase Superscript II (Invitrogen). Resulting cDNAs were PCR-amplified with primers 13 and 14 (Meng *et al.*, 1999b) by using AccuTaq LA DNA Polymerase (Sigma), cloned into pCR2.1 (Invitrogen), and recombinant plasmids transformed into JM109 cells (Promega). The cDNA products obtained by using primers 13 and 14 corresponded to nt 4373–4711 of the viral genome. We chose primers 13 and 14 for this analysis because they were designed based on the consensus sequence of multiple cDNA clones and because they could detect a wide spectrum of GRSPaV variants (Meng *et al.*, 1999b). After a quick screening by using PCR, recombinant plasmids were isolated by using a Qiagen Miniprep kit and sequenced on an ABI 373 sequencer using M13 forward and reverse primers.

In total, 52 clones from these ten 'St George' plants and six from 'BS5563' plants were sequenced and their sequences were analysed by using MegAlign (DNASTar). The results showed that the nucleotide sequences of all six clones derived from 'BS5563' plants were identical to one another and differed from that of GRSPaV-1 by 12 %. In contrast, the cDNA clones derived from 'St George' plants grouped into three clusters, SG1, SG2 and SG3, which differed from one another by 3.5–9.8 %. Among the ten plants assayed, seven were infected with SG1 alone, one with SG2 alone, one with SG1 and SG2, and the last (C1-2-3) with all three variants. Thus, SG1 was the predominant variant infecting the indicator 'St George' plants, at least among those that we assayed. Interestingly, all 23 cDNA clones from the 'St George' plants collected from British Columbia were identical and belonged to SG1 (data not shown).

In contrast, SG2, differing from SG1 by 9.8 %, was a minor variant, as it was detected in only two of the ten 'St George' plants assayed. SG3 was detected in 'St George' C1-2-3, the only plant in which all three variants were detected. GRSPaV-SG3 differed from SG1 by 5.3 % and from SG2 by 3.5 %. The frequency of GRSPaV-SG3 in this 'St George' plant was so low that it was represented by only one of the 11 clones sequenced.

These data suggested the following: (i) three new variants of GRSPaV were identified among the 'St George' plants assayed, with GRSPaV-SG1 being the predominant variant;

and (ii) the hybrid 'BS5563' was probably infected with a single GRSPaV variant (which we designated here GRSPaV-BS) that is distinct from GRSPaV-1 and from those derived from 'St George' plants. To unravel the relationship among GRSPaV-SG1, GRSPaV-BS and GRSPaV-1 at the genome level, we set out to sequence the entire genomes of GRSPaV-SG1 and GRSPaV-BS by using an RT-PCR-based stepwise approach (Fig. 1a). Again, the templates were dsRNAs isolated from 'St George' plants infected with GRSPaV-SG1 only or from 'BS5563' plants. Initial clones were obtained by using RT-PCR and primers derived from GRSPaV variants for which sequences were available. Gaps between the initial clones were then bridged by using variant-specific primers. Furthermore, two independent approaches were used to obtain the 5'-end genomic sequences. In the first approach, dsRNAs were polyadenylated, reverse-transcribed and cDNAs were PCR-amplified by using primers d(T)<sub>17</sub> and 28F3 (5'-CATCACGAC-TTGTCACAAACC-3'). Resulting RT-PCR products were cloned into pCR2.1 and sequenced. In the second approach, 5' rapid amplification of cDNA ends (RACE) was performed for GRSPaV-BS. Primer Race-3 (5'-GTGCTAC-CAAGCTGAGATC-3') was used in first-strand cDNA synthesis. cDNAs were purified by using a QIAquick PCR Purification kit (Qiagen) and C-tailed by using terminal deoxynucleotidyl transferase (Fermentas). They were then PCR-amplified by using d(G)<sub>14</sub> and virus-specific primers. Gel-purified PCR products were cloned and sequenced as described above. Clones obtained through both methods revealed identical sequences at the 5' terminus of the virus genome.

After assembling sequences of these overlapping cDNA clones, the complete genome sequences of both variants were determined. The genomes of GRSPaV-SG1 and GRSPaV-BS comprised 8725 and 8724 nt plus a poly(A) tail, respectively. Like GRSPaV-1, five ORFs were identified for both variants (Fig. 1a). The 5'-terminal non-coding regions (5' NCRs) of both variants comprised 60 nt and the 3' NCRs were 176 nt for GRSPaV-SG1 and 175 nt for GRSPaV-BS. Table 1 shows the genomic positions of the coding and non-coding regions of both variants.

At the genome level, GRSPaV-SG1 and GRSPaV-1 were related more closely, with 87.3 % identity. In contrast, both

**Fig. 1.** Strategies used to sequence the genomes of two new variants of GRSPaV and sequence comparison. (a) Schematic diagram showing the strategies used to sequence the entire genomes of GRSPaV-SG1 and GRSPaV-BS. The identical genome structure of both variants is shown in the middle. The overlapping cDNA clones used to assemble the genome sequences of GRSPaV-SG1 and GRSPaV-BS are depicted as solid lines above and below the genome structure, respectively. Names of the cDNA clones are given below the lines. MT, Methyltransferase; PRO, papain-like protease; HEL, helicase; RdRP, RNA-dependent RNA polymerase; CP, capsid protein. (b) Sequence alignment of the 3' NCRs of the three GRSPaV variants. (c) Alignment of partial amino acid sequences (aa 451–750) of the polypeptides deduced from ORF1 of the three GRSPaV variants, with the sequence of GRSPaV-1 shown as the top line. Identical amino acids are shown as dots. Note that the majority of differences were located in the region between aa 451 and 750, which is flanked by the putative MT and PRO domains. GRSPaV-SG1, the major GRSPaV variant detected in the indicator *V. rupestris* 'St George'; GRSPaV-BS, the GRSPaV variant sequenced from 'Bertille Seyve 5563', a French–American hybrid grapevine; GRSPaV-1, the first GRSPaV variant to be sequenced (Meng *et al.*, 1998).

**Table 1.** Sequence comparison of the three variants of GRSPaV

GRSPaV-SG1, the major variant detected in the indicator *V. rupestris* ‘St George’; GRSPaV-1, the first variant whose genome was previously sequenced (Meng *et al.*, 1998); GRSPaV-BS, the variant sequenced from ‘Bertille Seyve 5563’, a French–American hybrid grapevine. The results are shown as percentage identities of the entire genome sequences, their NCRs and individual ORFs. nt, Nucleotide sequence; aa, amino acid sequence; NA, not applicable.

Sequence (nucleotide position)	GRSPaV-SG1 vs GRSPaV-1		GRSPaV-BS vs GRSPaV-1		GRSPaV-SG1 vs GRSPaV-BS	
	nt	aa	nt	aa	nt	aa
Overall	87·3	NA	84·3	NA	83·9	NA
5' NCR (1–60)	98·3	NA	96·7	NA	98·3	NA
ORF1 (61–6546)	86·5	92·2	85·5	92·7	85·0	91·6
ORF2 (6577–7242)	87·2	93·2	78·5	86·9	78·2	86·5
ORF3 (7244–7597)	91·2	98·3	80·8	89·8	79·9	88·1
ORF4 (7518–7760)	91·4	88·9	83·1	88·9	84·0	86·4
ORF5 (7770–8549)	90·6	96·2	82·3	92·7	81·7	90·8
3' NCR (8550–end)	91·8	NA	84·0	NA	80·9	NA

variants were less similar to GRSPaV-BS, with 83·9 % identity between GRSPaV-SG1 and GRSPaV-BS and 84·3 % identity between GRSPaV-1 and GRSPaV-BS (Table 1). When homologous regions of all three variants were compared, levels of sequence similarity varied depending on the regions compared. For example, the 5' NCRs were most conserved, with 1–2 nt differences among the three variants (data not shown). This region is believed to contain regulatory sequences that are important for virus replication (Hull, 2002). This functional conservation may have posed constraints on mutations. In contrast, the 3' NCRs were more divergent, with nucleotide identities ranging from 80·9 to 91·8 % (Table 1). The majority of the differences were found in the region immediately downstream of the capsid protein (Fig. 1b).

Concerning the coding regions, sequence identities varied depending on individual ORFs. For example, when compared in their entirety, the ORF1s of the three variants were 85·0–86·5 % identical in nucleotide sequence and 91·6–92·7 % identical in amino acid sequence (Table 1). However, closer examination revealed that more than 50 % of the amino acid differences clustered in a region between aa 451 and 750 (Fig. 1c), which was flanked by the methyltransferase and protease domains of the polypeptide deduced from ORF1 (Meng & Gonsalves, 2003). Conceivably, this region may have been under less selective pressure during evolution than other regions. A similar trend was observed for nucleotide differences among these ORF1s.

ORF2 appeared to be less conserved. For example, the amino acid sequences of the ORF2 translation products of the three variants had identities of 93·2 % (between GRSPaV-SG1 and GRSPaV-1), 86·5 % (between GRSPaV-SG1 and GRSPaV-BS) and 86·9 % (between GRSPaV-1 and GRSPaV-BS). When GRSPaV-1 and GRSPaV-SG1 were compared with GRSPaV-BS, most of the differences were found in the C-terminal halves of the deduced polypeptides.

On the other hand, the capsid proteins of the three variants were more conserved, having amino acid identities ranging from 90·8 to 96·2 % (Table 1). Contrary to ORF2, most of the differences (75–80 %) were found in their N-terminal halves (data not shown). A similar trend held true when the nucleotide sequences of ORF5 were compared.

To determine whether GRSPaV-SG1 could induce RSP symptoms, an indicator indexing experiment was conducted. Graft inoculation was carried out in 1999 according to Martelli (1993), except that the inocula used were 4–6 cm long stem pieces instead of chip buds. Indicator ‘St George’ plants were obtained by rooting dormant cuttings from mother plants C1-2-8 (GRSPaV-negative) and C1-2-10 (GRSPaV-positive). Resulting indicator plants were grafted with inocula from the following source plants: ‘St George’ C1-2-8 (GRSPaV-negative), ‘St George’ C1-2-10 (GRSPaV-positive) and ‘Seyval’ (GRSPaV-positive). Five replicates were included for each of the rootstock/scion combinations. Additionally, 15 plants each of non-grafted ‘St George’ C1-2-8 and ‘St George’ C1-2-10 were included as negative controls (Table 2). Grafted and non-grafted indicator plants were maintained in the greenhouse to ensure successful virus transmission through callus formation at the grafting union. These plants were subsequently transplanted to, and maintained in, the field plot until June 2004, when all plants were removed from the soil and wood symptoms were observed.

The results of this indexing experiment are summarized in Table 2. All but one of the ten ‘St George’ plants inoculated with inocula from ‘Seyval’ produced typical RSP symptoms, three with medium and six with severe symptoms; the tenth plant died. Initial cloning work indicated that ‘Seyval’ was infected with a mixture of distinct GRSPaV variants (B. Meng, unpublished data). In contrast, RSP symptoms were not observed on any of the ‘St George’ plants that were uninoculated, nor on any of the ‘St George’ plants that were graft-inoculated, regardless of the infection status of the

**Table 2.** Results of biological indexing for symptom expression of GRSPaV variant SG-1 on the indicator *V. rupestris* 'St George'

Rootstock (GRSPaV status)	Scion (GRSPaV status)	Replicates	No symptoms	With symptoms
'St George' C1-2-08 (–)	Non-grafted	15	15	0
'St George' C1-2-08 (–)	C1-2-08 (–)	5	5	0
'St George' C1-2-08 (–)	C1-2-10 (+)	5	5	0
'St George' C1-2-08 (–)	'Seyval' (+)	5*	0	4
'St George' C1-2-10 (+)	Non-grafted	15	15	0
'St George' C1-2-10 (+)	C1-2-08 (–)	5	5	0
'St George' C1-2-10 (+)	C1-2-10 (+)	5	5	0
'St George' C1-2-10 (+)	'Seyval' (+)	5	0	5

\*One of the graft-inoculated 'St George' plants died during the indexing experiment and thus was excluded from the final recording of symptoms.

inoculum and the rootstock (Table 2). This result demonstrated that GRSPaV-SG1 did not induce RSP symptoms. Consequently, infection of 'St George' with SG1 should have had no or little impact on past indicator indexing results. It remains unclear, however, whether SG2 and SG3, which were detected at low frequencies in 'St George' indicator plants, would induce RSP symptoms.

It remains a mystery how the indicator 'St George' became infected with GRSPaV in the first place. As the indicator 'St George' plants from British Columbia, New York and Bari all tested positive for GRSPaV, and all of these plants presumably originated from the Foundation Plant Services of the University of California at Davis, CA, USA, infection of 'St George' by GRSPaV might have occurred in the original source plants. A possible scenario is that the mother plant(s) carried the virus to begin with and that, due to its asymptomatic nature, the virus has escaped detection ever since. Alternatively, the mother plant(s) may have been free of GRSPaV, but became infected later on as a result of transmission via an unknown insect vector or grafting.

GRSPaV has been detected in many countries where grapevines are grown (Zhang *et al.*, 1998; Meng *et al.*, 1999b; Nolasco *et al.*, 2000; Stewart & Nassuth, 2001; Tarnowski *et al.*, 2002; Dovas & Katis, 2003; Espinha *et al.*, 2003; Petrovic *et al.*, 2003). Despite its ubiquitous occurrence, the aetiological role of GRSPaV in RSP remains unclear. An ultimate solution to this enigma may be provided through the use of infectious cDNA clones. We have recently created such full-length cDNA clones and are currently testing their infectivity.

In summary, through sequence analysis of cDNA clones corresponding to a representative genomic region of the virus, we detected three new variants of GRSPaV in a sample of the indicator 'St George' plants, with GRSPaV-SG1 being the predominant variant. The genomes of GRSPaV-SG1 and another new variant from the grapevine hybrid 'BS5563' were sequenced, revealing a genome

structure identical to that of GRSPaV-1. Lastly, we demonstrated experimentally that infection of 'St George' plants with GRSPaV-SG1 is asymptomatic.

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